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Research Article

Comparative protection of two antigens (whole-cell and outer membrane vesicle) of *Yersinia ruckeri* in rainbow trout (*Oncorhynchus mykiss*)

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Abstract

Yersinia ruckeri (*Y. ruckeri*)-associated disease remains a problem in fish farming, despite widespread vaccine use. *Y. ruckeri* is still one of the main causes of economic loss in the aquaculture industry. In this study, we developed outer membrane vesicles (OMV) as a new antigen and compared its efficacy with the classical antigen (whole-cell bacteria). Rainbow trouts were immunized with *Y. ruckeri*-derived OMVs and formalin-inactivated whole-cell bacteria by immersion route. The fish were then subjected to an intraperitoneal challenge with live bacteria during 2-10 weeks post-immunization. Subsequently, relative percentage of survival (RPS), antibody titer, lysozyme serum activity, lymphocyte percent and kidney pathological signs were evaluated. The OMV morphology was confirmed by electron microscopy. OMV and whole-cell bacteria immunized fish had higher RPS relative to the control group, but whole-cell bacteria resulted in higher RPS than the OMV group. Moreover, in the whole-cell bacteria group antibody titer was higher than the control and OMV groups. Lymphocyte percentage and lysozyme level in the whole-cell bacteria group was increased compared to OMV and control groups. *Y. ruckeri's* pathological damage was observed in the control group, while only eosinophilic secretions were identified for both immunized groups. These findings proved the protection of *Y. ruckeri*-derived OMV against *Y. ruckeri*, but whole-cell bacteria in the immersion method had a better effect on disease protection.

Keywords: Rainbow trout, Outer membrane vesicles (OMVs), *Yersinia ruckeri*, Whole-cell bacteria, Immersion.

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Introduction

Vaccination is the best way to fight infectious diseases and it is a key tool for success in livestock and aquaculture industries. Vaccine use is now a cost-effective key factor in the development of the rainbow trout industry (Komar *et al.*, 2004).

Enteric Red Mouth (ERM) disease (Tobback *et al.*, 2007) is a systemic disease caused by *Y. ruckeri* that infects a wide variety of species, such as rainbow trout, salmon, carp, catfish and causes a high mortality rate in all stages of life (Tobback *et al.*, 2007; Guijarro *et al.*, 2018). Bacterial pathogenesis tends to occur in smaller fish and become chronic in adult fish. Infected fish are sometimes asymptomatic and sometimes show signs of encephalitis that may be misdiagnosed with viral infection (Guijarro *et al.*, 2018; Strøm *et al.*, 2018). Regarding bacterial pathogenesis, vaccination is the best way for disease control (Tkachenko *et al.*, 2016). The history of ERM vaccine goes back to USA in 1976 (Adams, 2019). Although formalin-inactivated whole-cell bacteria have been the main antigen for the vaccines since 1980s (Monte *et al.*, 2016). These vaccines did not have significant protection for several reasons, such as differences in bacterial serotypes (O1, O2, O5, O6, O7, O8) and biotypes (bt1 and bt2). So, we have outbreak reports annually (Akhlaghi and Sharifi Yazdi, 2008; Ormsby *et al.*, 2016; Tinsley *et al.*, 2011; Marana *et al.*, 2019; Wrobel *et al.*, 2019). It is, therefore, necessary to investigate new antigens' capability as vaccine candidates.

OMVs are parts of Gram-negative bacteria, which have the same components as bacterial cells. Based on OMVs' structure, they could transport toxins and virulence factors to host cells and stimulate the immune response (Toyofuku *et al.*, 2019). Studies showed that OMVs would train the immune system to fight against pathogens. Interestingly, it is shown that this immunization is induced without adjuvant use (Beikzadeh and Nikbakht Brujeni, 2018). OMVs are therefore a promising candidate for the development of vaccines against bacterial infection (Wang *et al.*, 2019). In fish vaccine technology, using OMVs as vaccine candidates is limited to a few studies on Francisellosis and Piscirickettsiosis in Zebrafish model (Pierson *et al.*, 2011; Lagos *et al.*, 2017; Tandberg *et al.*, 2017). Although several whole cell-killed vaccines are available to prevent the disease, particularly in the commercial form in Iran (Soltani *et al.*, 2014a, 2014b, 2016; Shafiei *et al.*, 2018), the bacteria are still leading to infection in rainbow trout and no study on the Yersiniosis-derived OMV antigen is conducted. This study aimed to compare whole-cell bacteria and bacteria-derived OMV as antigens in terms of protection against *Y. ruckeri* pathogenesis.

Materials and methods

Bacterial strain and growth condition

The *Y. ruckeri* used in this study was isolated during 2017-2018 from infected

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rainbow trout farms and approved by Persian Type Culture Collection (PTCC, accession no: MN382357, Iranian Biotype 1). Bacteria were cultured in Tryptic Soy Broth (TSB) for 24 hrs. at room temperature. Then bacterial culture was centrifuged at 10,000×g for 15 min to separate supernatant from bacterial pellet and stored in 4ºC until use.

OMV isolation and characterization

OMV isolation was done as described by Beikzadeh and Nikbakht Brujeni (2018). In brief, to concentrate high molecular weight proteins and remove low molecular weight proteins, supernatant was transferred to Crossflow system (Sartorius, Germany), Then, the retentate product was pelleted using a high-speed centrifuge (Refrigerated SIGMA 3-16K Centrifuge) at 20,000×g for 3h at 4°C. Isolated OMVs were resuspended in phosphate-buffered saline (PBS; pH 7.2), aliquoted and sorted at −80°C until use. To determine protein concentrations, the Warburg-Christian method was used to correct nucleic aside contamination and calculate only protein concentration (Shanmugam *et al.*, 2019).

Scanning electron microscopy

Scanning Electron Microscopy (SEM) was used to verify *Y. ruckeri*-OMV morphology. OMVs were fixed overnight in 5% paraformaldehyde. Then the sample was postfixed in 1% (wt/vol) osmium tetroxide for 2hr at room temperature. After dehydration through a serial ethanol grade, a fixed sample was covered by gold (Tobback, 2009).

Preparation of formalin-inactivated whole-cell bacteria

Y. ruckeri was recovered from diseased rainbow trout in Iranian farms during 2017-2018 and was used as an antigen according to Soltani *et al.* (2014a). A single colony of bacteria was inoculated into TSB (250 mL) at 25°C for 72 h. This culture was used to inoculate the bulk culture of 10 L. Then the culture was pelleted by centrifugation at 3000×*g* for 15 min, and then washed with sterile PBS, re-suspended in 1 L of PBS and was formalin-inactivated (0.5% Formaldehyde, Merck) at 4°C for 72 h. Bacterial cell count was done using OD600 and a final concentration of bacteria was adjusted on 4×10^9 colony forming units (CFU) mL^{-1}. The sterility of *Y. ruckeri* inactive antigen was confirmed by streaking bacterial samples into blood agar plates, 25°C for 72–96 h.

Experimental design and challenge protocol

The study population consisted of 1000 rainbow trout (*Oncorhynchus mykiss*) fish (40 g) obtained from a trout fish farm (Alborz Caspian farm, Alborz, Iran) that were randomly divided into three groups containing 250 fish per group (control group, OMV group and whole-cell bacteria group) (Soltani *et al.*, 2014b). Fish were kept in 3 tanks (temperature: 13±0.5°C, O_2: 8 mg/L, fed: 1% biomass using commercial pellet, tank volume: 350 L). To define

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the fish were free from *Y. ruckeri* infection before the experiment, sampling was taken from the kidney head and cultured in blood agar. For immunization of fish with whole-cell bacteria, formalin-inactivated *Y. ruckeri* biotype 1 (4×10^9 cell/mL) were administrated by immersion (the administration route with low stress for fish) in a concentration of 1:10 for 3 min; and for the OMV group, the *Y. ruckeri*-OMV (containing ≈2µg total protein) applied similar to the whole-cell group method. During 2, 4, 6, 8 and 10 weeks post-immunization 25 fish per group in 2 replicates were anesthetized with clove (*Syzygium aromaticum*) powder (0.2 g/L) and were administered with 6×10^6 CFU/mL of live bacteria by intraperitoneal (i.p.) route then quarantined to record the relative percentage of survival for 3 weeks (Monte et al., 2016). All fish were euthanized using an overdose of clove powder to obtain samples (blood and tissue). After the study, the remaining fish were euthanized by an overdose of clove powder (Fernandes *et al.*, 2017; Okey *et al.*, 2018).

Effectiveness of whole-cell bacteria and OMV

After the challenge, fish were monitored for 3 weeks and relative percentage of survival (RPS) on 2, 4, 6, 8 and 10 weeks post-immunization was calculated based on the below formula (Soltani *et al.*, 2014b):

RPS =1 - (% mortality of immunized fish / % mortality of unimmunized fish) × 100

Blood collection

Blood samples were obtained from caudal vein puncture (5 fish per group) at 2, 4, 6, 8 and 10 weeks after immunization. Blood samples were centrifuged at 2500×g for 10 min and serum was separated and stored at -80°C until use.

Antibody titer

The microplate agglutination method was used for the evaluation of antibody titer based on Altun *et al.* (2013) and Biller-Takahashi *et al.* (2014). Serum was obtained from samples of whole-cell bacteria and OMV immunized fish (5 fish per group before challenging with bacteria) at weeks 2, 4, 6, 8 and 10 and diluted with PBS on 1:1 (50 mL of PBS: 50 mL of serum) and was put into a microplate. Then, 50 mL of formalin-inactivated *Y. ruckeri* (10^7 CFU/mL) as antigen was added to each well. The wells that appear fuzzy egg were considered as positive reaction. The titer was measured as the reciprocal of the maximum dilution ($\log_2$) of serum showing complete agglutination of the bacterial cells.

Lysozyme activity assay

Lysozyme serum activity was evaluated according to Soltani *et al.* (2014b). In brief, 1.75 mL of *Micrococcus lysodeikticus* was diluted in PBS at pH 6.2 and added to 250-µl pooled serum

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samples. The optical density of samples was measured at 450 nm wavelength immediately and after 3 min. The standard curve from the different concentrations of chicken egg white lysozyme was used to assess serum samples lysozyme activity.

Lymphocyte population changes

The total lymphocyte of blood samples was counted by the Giemsa staining method and the results were reported in percentage (Ataeimehr *et al.*, 2014).

Histopathology

Kidney samples from each group were fixed by formalin, embedded in paraffin, and sectioned with a microtome to obtain 4 µm thick sections. Sections were put on slides and stained with hematoxylin and eosin (H&E) (Slaoui and Fiette, 2011).

Statistical analysis

Statistical analysis was done using GraphPad Prism 8 software. ANOVA and Tukey post hoc tests were used for group comparison and significance was considered as $p<0.05$. Data are represented as mean±standard error. The RPS results were analyzed using 2-way ANOVA followed by Tukey's multiple comparison test.

Results

Morphology of Y. ruckeri-derived OMVs

Scanning electron microscopy confirmed the spherical shape of *Y. ruckeri*-derived OMVs in different sizes from 20 to 40 nm. Based on the obtained picture, the supernatant was transferred from the crossflow system using three filter types and removed bacterial residues in the final product (Fig. 1).

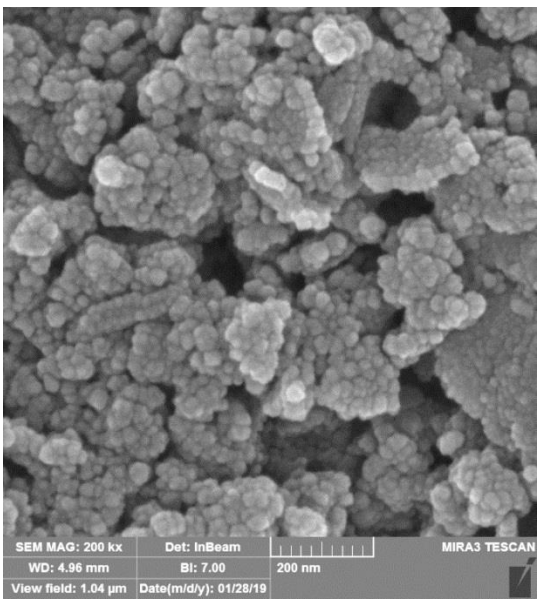


Figure 1: OMV morphology. Electron micrograph of *Y. ruckeri*-derived OMVs. To confirm the purity of OMV isolation from *Y. ruckeri* supernatant culture, they were visualized by scanning electron microscopy. The spherical shape of OMVs without bacterial debris is clear. The image was obtained by MIRA 3TESCAN. Bar=200 nm.

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Effectiveness of whole-cell bacteria versus OMVs against Yersinia ruckeri

The whole-cell bacteria and OMV significantly protected rainbow trout challenged with *Y. ruckeri* (6×10^6 CFU/mL, Fig. 2). The RPS in fish immunized with whole-cell bacteria of 84%, 84%, 80%, 78% and 78% were higher in comparison to OMV (54%, 45%, 41%, 40% and 38%) and control groups (2%, 1%, 1%, 1% and 1%) on 2, 4, 6, 8 and10 weeks post-challenge, respectively (p=0.0001). However, regarding evaluation of OMV effectiveness, the immunized fish had higher survival rate when compared to the control group (p=0.0001).

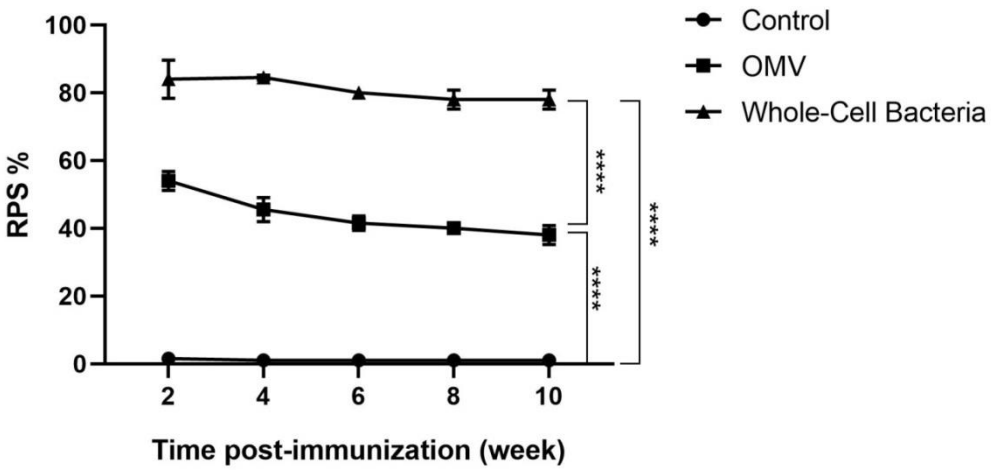


Figure 2: Relative percent of survival (RPS) of fish immunized with OMV and whole-cell bacteria during weeks 2, 4, 6, 8 and 10 post-immunization, fish were challenged with live *Y. ruckeri* (6×10^6 CFU/mL) by i.p. route. OMV and whole-cell bacteria groups had a high percentage of survival. However, fish immunization with whole-cell bacteria was more efficient. Asterisks (*) represent *P* values between groups (** p<0.001). Error bars show standard error.**

Antibody titer

Evaluation of antibody titer showed no significant difference between whole-cell bacteria and OMV immunized groups after 2 weeks post-immunization. While at weeks 4, 6, 8 and 10 the antibody level of the whole-cell bacteria group was significantly increased in comparison to that of the control and OMV groups (p<0.05). Conversely, the antibody titer in OMV immunized group was reduced in 10 weeks post-immunization compared to that in weeks 4 and 6 (Fig. 3).

Serum lysozyme level

The lysozyme serum level as one factor of innate immunity in fish immunized with whole-cell bacteria and OMV in comparison to the control group showed no significant difference in 2 weeks post-immunization (p>0.05). While at week 4, the lysozyme level in the whole-cell bacteria group increased in comparison to the OMV group (p=0.0498). This significant change continued at weeks 6 (p=0.0045), 8 (p=0.0296) and 10 (p=0.0454) post-immunization. Also, at weeks 6, 8 and

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10 serum lysozyme level in the control group was generally lower (p<0.05) than that in whole-cell bacteria and OMV groups (Fig. 4).

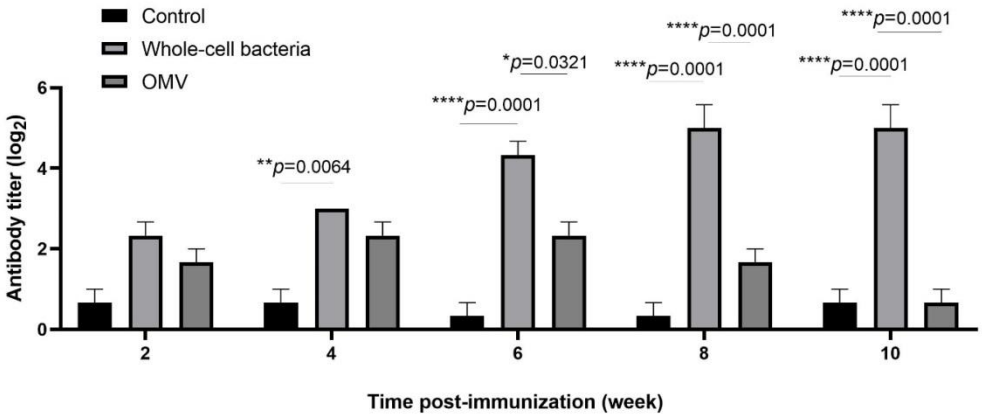


Figure 3: Serum antibody titer of fish immunized with OMV and whole-cell bacteria during weeks 2, 4, 6, 8 and 10 post-immunization. The fish were immunized with whole-cell bacteria had high antibody levels compared to the control and OMV groups (*P*<0.05). Error bars show standard error.

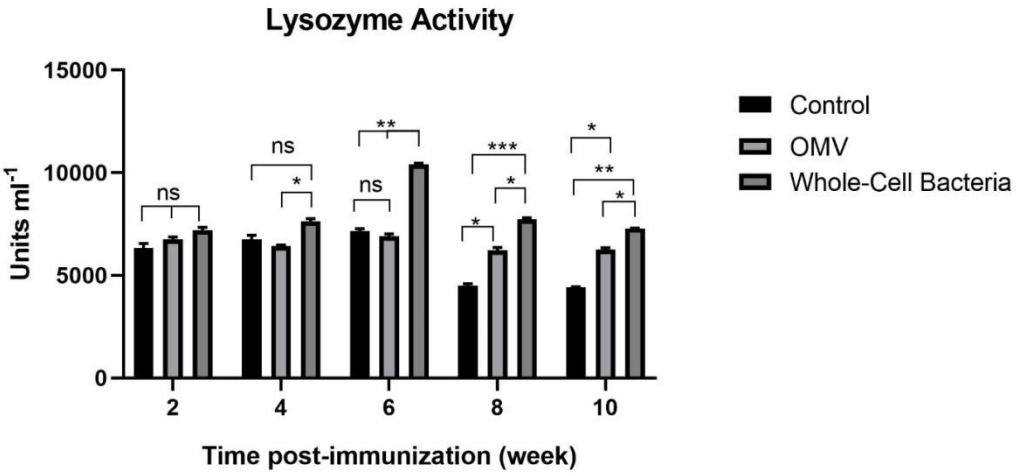


Figure 4: Serum lysozyme activity evaluation showed that the fish immunized with whole-cell bacteria had higher activity than the fish of OMV and control groups. Comparison of all groups was performed by 2-way ANOVA-Tukey's multiple comparison test. Asterisks (*) represent *P* values between groups. **P*=0.0498, ***P*=0.0045, ****P*=0.0005, ns=*P*>0.05. Error bars show standard error.

Blood lymphocyte percent

In general, immunization with whole-cell bacteria and OMVs significantly increase the lymphocyte population over weeks 2, 4, 6, 8 and 10 compared to the control group (p<0.05). However, our results proved whole-cell bacteria had more potential for increasing the lymphocyte population (p<0.05) than the OMVs (Fig. 5).

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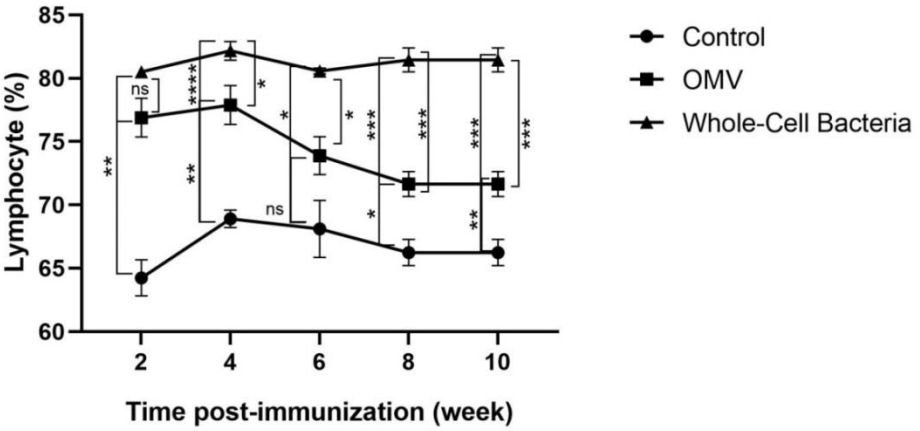


Figure 5: Blood lymphocyte percent during weeks 2, 4, 6, 8 and 10 post-immunizations. Fish immunization with whole-cell bacteria had a higher lymphocyte population in comparison to fish of OMV and control groups. Comparison of all groups was performed by 2-way ANOVA-Tukey's multiple comparison test. Asterisks (*) represent *P* values between groups. *******P*=0.0001, ******P*= 0.0005, *****P*=0.0035, ****P*=0.0186. Error bars show standard error.

Histopathology findings

Kidney histopathology showed that after being challenged with *Y. ruckeri*, hyperemia was evident in the control group. However, in the whole-cell bacteria and OMV immunized groups, eosinophilic secretions in proximal urinary tubules were observed (Fig. 6).

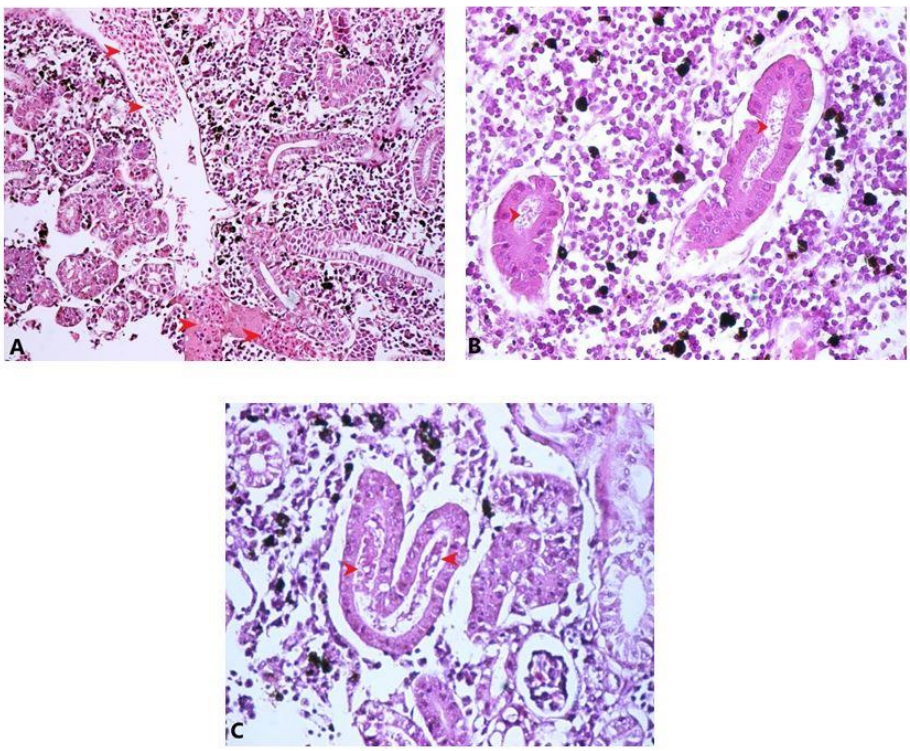


Figure 6: Gross pathology of rainbow trout kidney (H&E, X200). A: hyperemia in the control group (red arrowheads, X100). B and C: vaccinated groups: whole-cell bacteria and OMV, eosinophilic secretions (red arrowheads).

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Discussion

Vaccination is one of the most important ways to prevent diseases (Van Muiswinkel, 2008). Yersiniosis in fish is a worldwide disease and is not limited to Iran (Kumar *et al.*, 2015). However, many questions remain about pathogenesis, immune response and prevention of disease. The high prevalence of the disease still questions the effectiveness of the current vaccines (Kumar *et al.*, 2015). In Iran, a study carried out to evaluate the killed whole-cell bacteria as a vaccine by the immersion method, confirms the efficacy of the vaccine in preventing yersiniosis (Soltani *et al*., 2014a, 2014b). Another research was also performed to use Montanide adjuvant with the vaccine and the findings showed an increase in immunity and fish survival during one-time vaccine use (Soltani *et al*., 2016). In the following work by Shafiei *et al.* (2018) use of adjuvant G2 also improved both the efficacy and potency of the vaccine towards *Yersinia* septicemia in rainbow trout. However, several factors are identified to improve the efficacy of vaccines and use of accompanying substances to make vaccines more effective, including immune stimulants (Siwicki *et al.*, 2001), adjuvants, etc. (Soltani *et al.*, 2016; Shafiei *et al.*, 2018). OMVs can be regarded as non-proliferating bodies that are naturally produced by Gram-negative bacteria and contain excellent immune-stimulating compounds. OMVs usually include phospholipids, lipopolysaccharides (LPS), outer membrane proteins and components trapped in the periplasmic space (Schwechheimer and Kuehn, 2015). There are now several studies that proved the interaction of OMVs with the innate and adaptive immune system (Wang *et al.*, 2019; Gilmore *et al.*, 2021). CsgG, CsgA, TamA/YtfN, LptD, OmpW, Phosphate A1, OmpX, PilV, GlpC and ShuA are the outer membrane proteins that have a putative role in *Y. ruckeri* virulence (Ormsby *et al.*, 2019). It would be possible that these proteins and other components of OMVs activate TLRs as the first line of innate immune response (Mancini *et al.*, 2020). Furthermore, these nano-vesicles induce the expression of costimulatory molecules and receptors on dendritic cells that result in T cell activation and antibody response (Tan *et al.*, 2018).

We investigated the use of OMVs as an antigen to further the vaccine efficacy. The harvested OMVs from *Y. ruckeri* culture by several filtration techniques had similar morphology, range size and purity with that of other studies (Beikzadeh and Nikbakht Brujeni, 2018). To assess the efficacy of OMV and whole-cell antigens, we used the immersion route as a simple immunized method (Osman *et al.*, 2009). Results of relative survival percent evaluation showed that the whole-cell immunized group had maximum efficacy compared to OMV immunized and control groups. The effectiveness of whole-cell antigen in antibody production was confirmed in studies carried out by Soltani *et al.* (2014a, 2014b, 2016). In the present study, there was a direct relationship

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between relative survival percent and antibody level for the whole-cell immunized group. While previous studies on other species (human and animal) confirmed that the OMVs could be vaccine candidates instead of whole-cell bacteria (Brudal *et al*., 2015; van der Pol *et al.*, 2015), this defect in OMV protection is probably due to the route of immunization (Immersion versus injection); in the immersion method, vaccine antigens are diluted in water. However, OMV immunized group had significant protection compared to the control group (p<0.05).

To date, in rainbow trout, only two studies on the use of OMVs for *Francisella* and *Piscirickettsia* showed that OMVs are capable of immunizing Zebrafish against bacteria. (Lagos *et al*., 2017; Tandberg, 2018). According to our results and models from human studies, there is a promise in the potential of OMV immunogenicity for the production of a new generation of the acellular vaccine.

Previous studies proved a significant increase in the level of some innate immune factors including lysozyme and lymphocyte population during fish immersion-vaccinated using commercial vaccine (Aqua VacTMERM vet) or *Y. ruckeri* bacterin (Raida *et al*., 2011; Soltani *et al.*, 2014b). In the present study, the serum lysozyme level and lymphocyte percent were higher for the full mass immunized group than the other groups, which may indicate a better immune stimulation and efficacy of the whole-cell bacteria. On the other hand, kidney hemorrhage and telangiectasia in the control group after being challenged were reported by other studies as well (Mahjoor and Akhlaghi, 2012). Overall, our histopathology findings from the whole-cell and OMV groups showed mild inflammation with eosinophilic secretions which confirmed successful immunization of fish.

The OMV antigen has the potential to produce immunity against yersiniosis disease. However, according to our results, immunization with whole-cell bacteria was more efficient in the immersion route. This issue was related to the route of immunization, it seems the immersion route causes OMVs more diluted, while in previous studies i.p. route was used for immunization. On the other hand, another reason for low OMV efficacy is that while OMVs have bacterial antigenic properties, they may not carry all immunogenic antigens. Finally, our results confirmed that OMV immunogenicity could contribute to the development of fish vaccine technology, but OMV vaccines are in the early stage in fish vaccinology and require further study in particular on OMV antigenic contents and possible potential for cross-protection between biotypes.

Research limitation

- Fish immunization with OMV protects them from the disease, but not the same as the whole-cell bacteria vaccine.
- The immersion route is not suitable for OMV immunization.
- Research on fish vaccines usually focused on whole-cell bacteria, while

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OMVs could be a new vaccine candidate with low side effects and high immunity. The injection route is recommended for further studies.

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